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Female Invasive Crayfish *Faxonius Rusticus* Prefer Pheromones of Conspecific Males During the Breeding Season

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Female invasive crayfish *Faxonius rusticus* prefer pheromones of conspecific males during the breeding season.

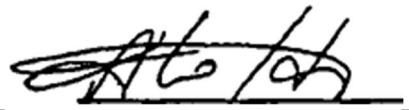
By

Elizabeth Luscavage

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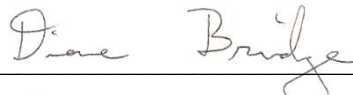
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Abstract

The invasive crayfish *Faxonius rusticus* has invaded most watersheds in Pennsylvania, spreading throughout the Northeastern United States. Existing methods to regulate *F. rusticus* populations have been ineffective. Because crayfish communicate chemically through pheromones, understanding what information these pheromones carry can help improve trapping methods. The aim of this study was to determine how *F. rusticus* respond to chemical signals produced by same and opposite sex conspecifics during breeding and non-breeding seasons. Previous studies in our lab have shown that when presented with a choice of either a pheromone or a water control, during both breeding and nonbreeding season, female *F. rusticus* are attracted to pheromones produced by males. Males however avoid signals of conspecific males, and do not show preference for female signals. In this study, we utilized a Y-maze to present *F. rusticus* with a choice of male or female conspecific pheromone simultaneously, during breeding and nonbreeding seasons. Our findings show that females prefer male conspecific pheromones during breeding season, but do not exhibit a preference during the non-breeding season. Males *F. rusticus* entered the arm of the maze with female pheromones more often during the breeding season, but that response was not statistically significant. In conclusion, females showed a preference for opposite-sex conspecific pheromones and avoided same-sex pheromones during the breeding season, but neither males nor females showed a preference for either pheromone during non-breeding seasons.

Introduction

Faxonius rusticus (*F. rusticus*) are an invasive crayfish species in Pennsylvania and through most northeastern states of the country. Native to the Ohio River Valley, *F. rusticus* have expanded into other states through hitchhiking on shipping containers, and being used as bait (Hill, 1999). They migrated outward to invade rivers and creeks, including Delaware, Potomac, Schuylkill, and Susquehanna (Kuhlman, 2007; Hill, 1999). *F. rusticus* has spread throughout northern and southern Pennsylvania becoming one of the dominant species in most watersheds. In a crayfish sampling study conducted in northern and southern PA, Lieb et al., (2011) found that invasive species *Cambarus bartonii*, *F. rusticus*, and *O. limosus* represented 76% of the total catch with *F. rusticus* making up 25% of the catch. Several factors affect the severity of *F. rusticus*' impact, including their higher consumption rates (Morse, 2013), decreased susceptibility to fish predation (Kuhlmann et al., 2008), and greater growth rate that allows them to attain larger carapace and chelae size than native crayfish counterparts (Hill et al., 1993). In addition, *F. rusticus* are more aggressive than most native crustaceans, giving them a competitive advantage over access to resources (Vorburger, 1999). These environmental and adaptive advantages have allowed *F. rusticus* to out-compete native fish and /crayfish species for food (GSID, 2010).

Current methods to decrease invasive species populations, such as pesticides, pond drainages, and introduction of fish predators have been ineffective and harmful to native species and the environment (Gherardi, 2011). Trapping is not species-specific, causing capture of native and invasive species (Hein, 2006; Hein, 2007). Therefore, the only way to decrease invasive crayfish species populations, without damaging the environment and killing native species, is through manual capture.

Crustaceans utilize chemical signals for communicating social status, territoriality, and social status, and reproductive state (Atema, 2007). These chemical signals are thought to be contained in the urine of the crayfish (Thiel and Brethaupt, 2011). The urine is released from the nephropores, in the head region and is then propelled by fan organs distributed around the mouth, opening below the major antennules and the nephropores (Breithaupt, 2001). During agonistic interactions highest urine concentrations were found near the antennular chemoreceptors, which likely pick up the signals sent by the opponents (Breithaupt, 2010).

During agonistic interactions, opponents release and direct urine towards each other. Winners release more urine than losers, (Breithaupt and Eger, 2002). Blocking the nephropores leads to a significant increase in fight duration between size-matched males of *Faxonius rusticus* (Zulandt Schneider et al. 2001), suggesting that urine communicates social status and fighting ability.

Crayfish pheromones could potentially be used as a more specific baited trapping method. Currently not much is known about the chemical composition of these pheromones. A study by Stebbing determined the size of the adult female *P. leniusculus* crayfish's pheromone to be about 10,000 Da (Stebbing, 2005). Previous studies examining crayfish ability to discriminate between sex pheromones have yielded contradictory results. Some studies have shown that male crayfish can distinguish between conspecific male from female odors (Aquiloni, 2008; Breithaupt, 2008), whilst others have reported no discrimination at all (Thorp & Ammerman, 1978; Itagaki & Thorp, 1981). Previous studies in our lab found that female *F. rusticus* can distinguish male pheromones from a water control, but do not exhibit avoidance or attraction to signals produced by conspecific females. Males avoided signals produced by conspecific males, but did not exhibit a differential response towards female signals (Stonecipher et al., 2017; Kalmbacher and Goldina, 2018).

In this study we aimed to assess if male and female *F. rusticus* can distinguish between male and female pheromones of conspecifics and if this ability changes during breeding and nonbreeding seasons. If *F. rusticus* were to show a preference for opposite or same sex conspecifics, knowledge of pheromone preferences could be used for development of baited traps or repulsion barriers, which can improve trapping efficiency.

Materials and methods

1. Animal Collection and Holding

Male and female *Faxonius rusticus* were collected from Conoy creek in Elizabethtown, PA, USA between the months of May and October in years 2017-2020. Animals were housed in a light (12L:12D cycle) and temperature (27.25 °C) controlled aquarium facility. Animals were kept in mixed-sex groups in plastic troughs (105 cm X 72 cm X 30 cm), filled with well water. Animal numbers varied based on collecting season, ranging between 5 and 20 animals per trough. Each trough was lined with gravel and had rocks, shells, petrified wood, and

PVC shelters to mimic the natural streams as much as possible. Two air stones aerated and circulated water in the troughs. Partial water changes were conducted regularly, once a month. Animals were fed algae pellets (Aquatic foods inc) twice a week. All individuals were sexually mature, as identified by the presence of calcified ovipositors in males and major chelae size in females (chelae ≤ 11 mm).

2. Isolation procedures

Prior to behavior experiments, all animals were socially isolated in plastic 1.5 liter containers (29.85 cm x 19.68 cm x 20.32cm) for 7-9 days. The containers were filled with well water and aerated with an airstone. Each container also had 1 PVC shelter and a mesh bottom secured with 4 suction cups on each corner of the tank to help maintain water clarity. Three of the side walls of the containers were painted with black paint, to ensure visual isolation between the animals. Crayfish were randomly selected from the communal troughs and placed in the numbered isolation containers. Before placement in the containers: sex, carapace length, and weight of each individual was recorded. During the isolation period animals were not fed. At the end of the isolation period, water from the isolation containers was collected, filtered, and used as part of the pheromone stock for discrimination tests, while animals were tested using the discrimination tests described below. After each test, all containers were cleaned with detergent and 70% ethanol. While each crayfish was only tested in the discrimination test once, it might have been used for stock collection multiple times. Once an individual has been used for a behavioral experiment, it was placed in a separate trough to ensure that it was not reused.

3. Pheromone stock collection

For each experiment, two pheromone solutions were created, male and female. The pheromone stocks were further divided based on the season when they were collected. Stock collected between June and September was considered Breeding, and stock collected between the months of October- January was Non-breeding. These solutions were used during the discrimination experiment described below. Pheromones for adult female and male *F. rusticus* crayfish were obtained following the social isolation protocol described above. Water from each isolation container was filtered through a 150 nm filter paper, placed in

a 6 ml sealable bag, and frozen to be used as pheromone stock. On the day of the experiment the stocks from three different individuals of the same sex were thawed, combined, and diluted with deionized water at 50% concentration. This concentration elicited the most robust behavioral response in previous studies (Henry et al, 2018). A total volume of 12,000 ml was then placed in the respective containers connected to the Y-maze.

4. Y-Maze Design and Validation

A flow through Y-maze (1.5cm x 0.3cm x 0.3cm) was designed to assess attraction or avoidance responses to different chemical signals. Water was pumped by two MasterFlex® I/P Pump Drives Model 77410 with I/P 88 tubing from two separate 121L containers into their respective arms of the maze at a inflow rate of 2.4L/min per arm. Water level was maintained at a depth of 12.7cm by keeping outflow rate of 2.45L/min.

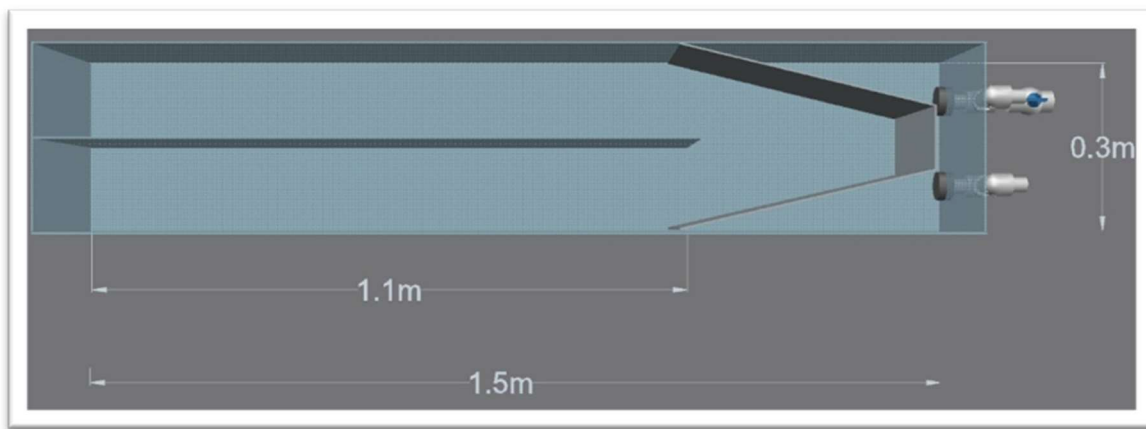


Figure 1: Experimental arena dimensions. View from top.

The body of the tank was separated into halves by a 1.1 m long plexiglass barrier. Each half-received water from a separate pump, allowing simultaneous delivery of two different chemical sources to the focal crayfish placed downstream of the maze. Dye trials were conducted in order to determine the speed at which the water travelled and to make sure that there was no back-flow in the arms or leakage between the arms. It was found that the water travelled at a speed of 2 cm/s and reached the end of the arms in approximately 1.5 minutes. At the end of the trough opposite the divider, a V-barrier was placed to entice the crayfish to move towards the arms of

the maze. A removable gate was placed at the opening of the V-barrier during acclimation periods to prevent the individual from exploring the arms (figure 2).

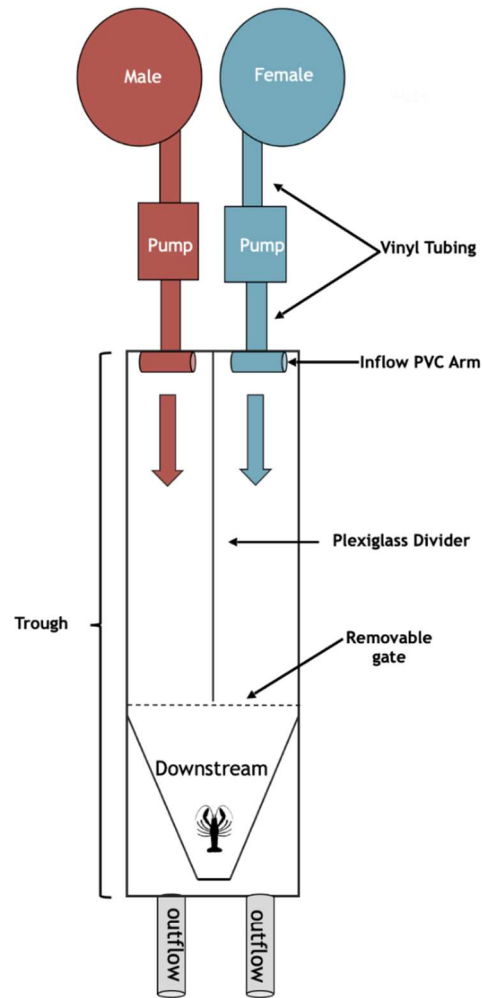


Figure 2: Experimental setup to assess pheromone preference. Pheromones from males and females were pumped into the arms of the maze. Crayfish was placed downstream of the Y-maze, acclimated for 15 minutes while being separated from the arms with a removable gate.

5. Experimental Design

The experiments and pheromone stock collection were conducted during the breeding (June – September) and non-breeding (October – January) seasons. To ensure absence of side bias, pheromone placement within the arms of the maze was randomized. Previous studies in the

lab using the same experimental protocol have shown that crayfish can distinguish between pheromones and a water control (Kalmbacher and Goldina, 2018), and was thus not repeated in this study. A crayfish was randomly selected from the social isolation tank, and its sex, weight, total length, and lengths of major chealae length recorded 7-9 days prior to testing (Females, n=26, average weight = 19.12g, total length = 70.92mm, chelae length =35.5mm; Males, n=24 weight =24.52g, total length = 74.96mm, chelae length =30.06mm). On the day of the behavior test, the crayfish was placed behind a mesh screen towards the front of the Y-maze for 15 min to acclimate. Two buckets were filled with 12,000 mL of different pheromones connected to a Y-maze that was filled with 50 L of water (figure 2). Pheromone stocks were then pumped through the arms of the maze for 223 seconds at the rate of 2.4 liters per minute. This time has been shown to be sufficient for the pheromones to reach the end of the maze arms, where the crayfish placed for discrimination trials (Henry and Goldina, 2018). After that, the mesh screen was removed, and the movement of the crayfish was recorded using an Akaso action camera for 20 minutes. Distance traveled in the maze, which arm was selected, and amount of time spent in each arm was recorded.

The Y-Maze and buckets containing pheromone stocks were cleaned with 70% bleach and ethanol after each trial.

6. Statistical analysis

Variables recorded included time spent in each arm, distance traveled in each arm, number of turns in each arm. Within each sex, side-bias was assessed to make sure that crayfish were not choosing a particular arm of the maze simply based on location. A one-tailed t-test compared the frequency of entries into each arm, using the same pheromone. Absence of side bias was assumed when p-value was greater than 0.05, showing no significant difference between arms when pheromone type is held constant. A one-tailed t-test was used to assess whether crayfish spent more time in the same-sex or opposite sex pheromones, walked more within a given pheromone arm, and made more turns. A p-value was then calculated via a one-tailed t test for every variable, and significance criterion was set to $\alpha=0.05$.

Results

Overall, female and male crayfish spent more time in the arms containing opposite-sex pheromones (Fig. 3a; females $p=0.02$; Fig. 3b; males $p=0.06$). Within the breeding season, females maintained their preference for male pheromones and spent more time in that arm (Fig. 4A, $p<0.01$), while males did not exhibit a preference (Fig. 4B, $p=0.11$). During the non-breeding season, amount of time spent in either arm was not significantly different for either sex (Fig. 5A, females $p=0.12$; Fig. 5B, males, $p=0.09$). During both breeding and nonbreeding seasons, females turned more in the male pheromone arm than the female (Fig. 6A $p=0.02$).

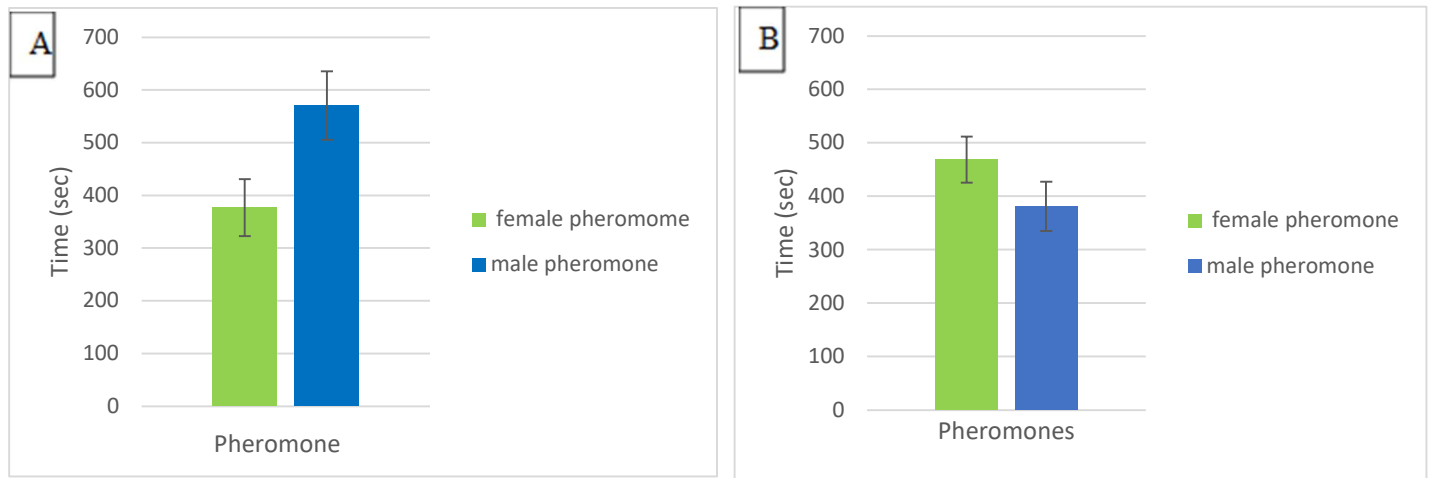


Figure 3: Average time spent in the arm containing male and female pheromones. A. females spend more time in the arm with male pheromone ($p=0.02$), and B. Males spend more time in the arm containing female pheromones ($p=0.06$)

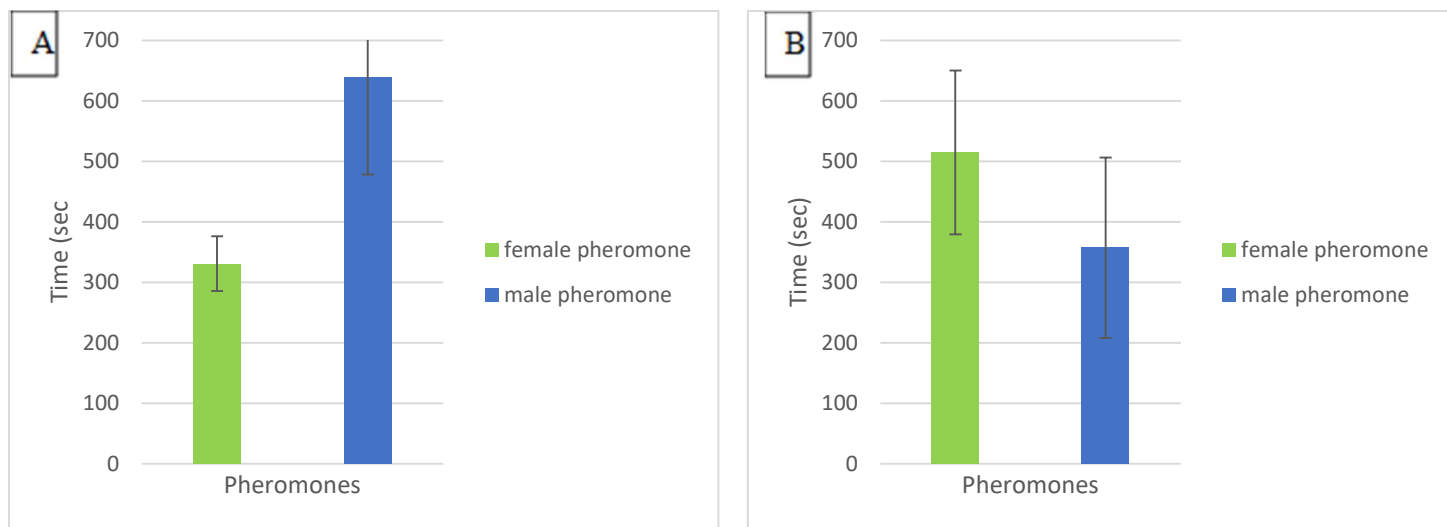


Figure 4: During the breeding season (A) females spend more time in the arm containing male pheromone ($p < 0.01$), while (B) Males did not show preference ($p = 0.11$)

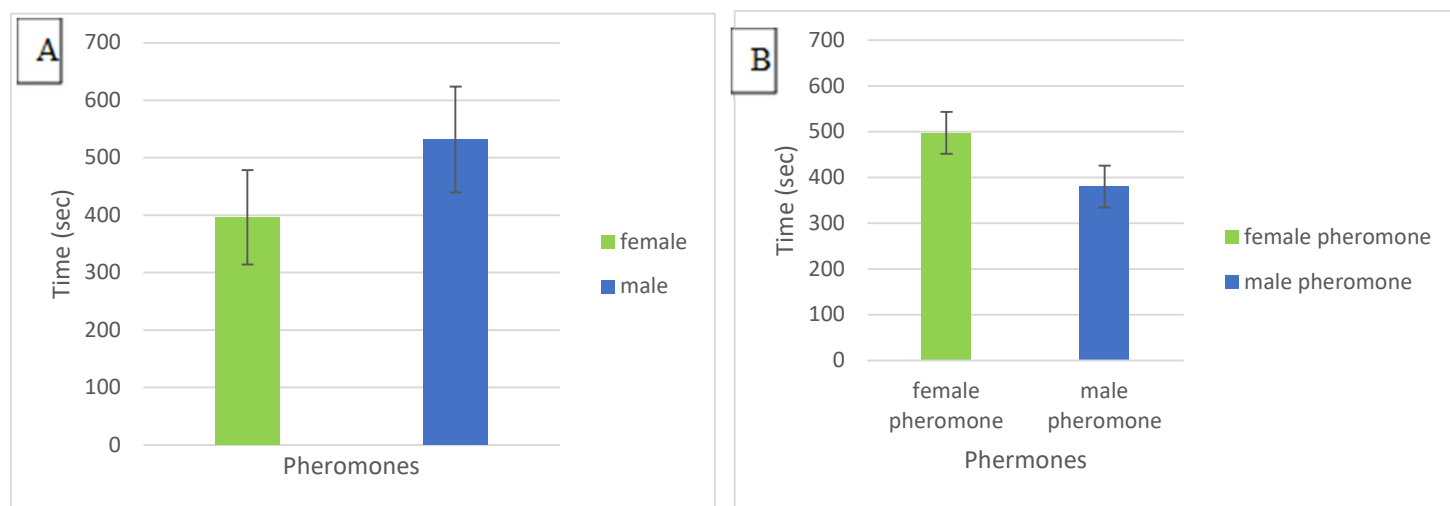


Figure 5: During the non-breeding season, neither (A) females ($p = 0.12$) nor (B) males ($p = 0.09$) exhibited a preference for either pheromone. ($p < 0.01$), while (b) males did not show a preference ($p = 0.11$).

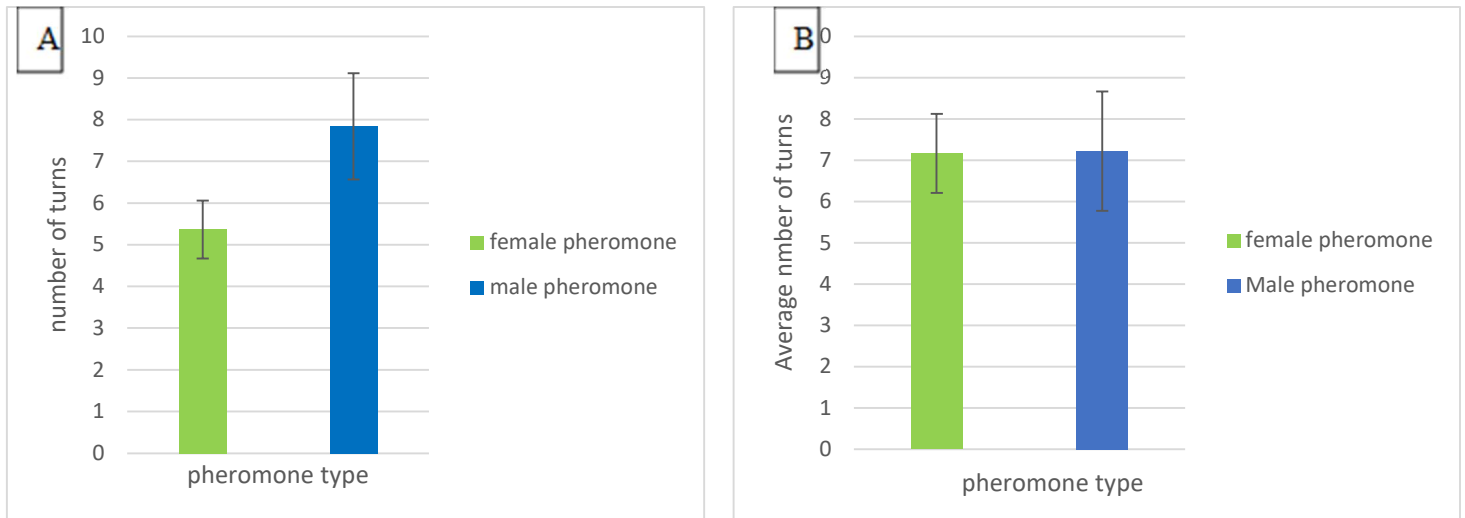


Figure 6: During both breeding and nonbreeding season, (A) females turned more in the male pheromone arm ($p=0.02$). (B) males did not show a preference ($p=0.27$)

Discussion

Our result indicated that female *F. rusticus* are attracted to male *F. rusticus* pheromones during breeding season, but not during the nonbreeding season. Other studies conducted on different species of invasive crayfish were contradictory to our finding. A study by Aquiloni et al., in 2008 supported that the invasive species *Procambarus clarkii* responded to the opposite sex pheromones differently depending on their sex. The males relied on olfaction alone for sex identification, while females required the combination of olfaction and vision to do so. This study contradicts these findings. We found that the female *F. rusticus* spent more time in the male pheromone arm indicating the female olfactory information is sufficient to seek out the opposite sex. Male crayfish *Pacifastacus leniusculus* mount a female dummy for significantly longer after the release of female urine in comparison to male odors and the water control (Berry and Breithaupt, 2008). These contradictory results suggest that response to pheromones is species-specific and developing baited traps to regulate invasive crustacean populations will require understanding the response of the target species.

The results of this study also showed that male *F. rusticus* show no preference for female *F. rusticus* pheromones during nonbreeding season (fig. 5), but there was a trend of them showing interest in female pheromones during breeding season (fig. 4). While it is unknown

what changes occur to the crayfish's pheromones during breeding season it is known that some physiological changes do occur. During the breeding season of the different crayfish species, there are physiological changes that do occur. The breeding season for *F. rusticus* specifically is late summer (June-July) and early fall months (August to early September) (Berrill, 1984). It was noted that some cambarids, *Orconectes propinquus*, *Orconectes limosus*, and *Faxonius rusticus* exhibit a cyclic alternation of form that is related to the breeding season. They found that in the summer months cambarids males molt to a non-breeding stage and then molt back again 8–10 weeks later. They also noted that during the northern summer *Orconectes propinquus* males molt twice, firstly from form I (breeding form, had longer and wider chelae than females and form II males) to form II (non-breeding form, had chelae of intermediate size) in mid-June, and then back to form I again in August (McClay, 2016). Urine from females during a breeding season has been shown to elicit an increase in mounting behavior, but not an increase in other breeding behaviors (Berry and Breithaupt, 2008). These studies support that there are both physiological and behavioral changes occurring during breeding season, suggesting that the pheromones produced by crustaceans vary seasonally to reflect the physiological changes.

Since it has been supported that male pheromones attract female crayfish during breeding season, and the crayfish themselves undergo physiological changes during these breeding seasons, there is a possibility that these male pheromones could be implemented in baited traps.

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